(51) International Patent Classification 6 : A01K 67/027, C12N 15/63	A1	(11) International Publication Number: WO 95/22249
		(43) International Publication Date: 24 August 1995 (24.08.95)

(54) Title: TRANSGENIC FIBRINOGEN

(57) Abstract

    Transgenic non-human animals, capable of expressing a heterologous gene for human or other recombinant fibrinogen protein or subunit chain polypeptides thereof or modified fibrinogens in mammary glands of the animals and secreting the expressed product into a body fluid, are provided, as are methods of producing recombinant fibrinogens, subunit chains polypeptides thereof and modified fibrinogens, and fibrinogens fusion proteins in such animals.

TRANSGENIC FIBRINOGEN

BACKGROUND OF THE INVENTION

Field of the Invention

This application relates in general to the use of
transgenic animals to produce therapeutically useful
amounts of clinically important proteins. More
particularly, this application relates to the production
in transgenic animals of clinically useful quantities of
the blood clotting protein, fibrinogen ("FIB").

Description of the Background Art

The ultimate event in the blood clotting cascade is
the thrombin-catalyzed conversion of FIB (Mr=340,000) to
fibrin (Mr=329,000), the latter forming the fibrin clot.
FIB deficiency is generally transmitted as an autosomal
recessive trait and may manifest as a complete or partial
absence of FIB from the blood plasma. Clinically, the
disease resembles moderate or mild hemophilia. Congenital
fibrinogen abnormality may be due to the hereditary
synthesis of structurally or functionally abnormal
molecules, as in Vlissingen, Ijmuiden and Nijmegen
fibrinogens. An acquired deficiency of this protein may
occur as the result of impaired hepatic synthesis of the
protein as, for example, in hepatitis or hepatic
necrosis, or to accelerated destruction of the protein
caused, for example, by increased blood proteolytic
activity.

Control of bleeding in such patients is currently
achieved by transfusion of FIB contained in freshly-
frozen human plasma or in concentrates of the protein
isolated from donor blood. While these replacement
therapies are generally effective, they place patients at
risk for virus-transmissible diseases such as hepatitis
or AIDS. Although this risk has been greatly reduced by
inactivating such viruses with heat or organic solvents,
such preparations have greatly increased the cost of
treatment, and are not risk free. There is thus a
critical need for a source of this protein alternate to
human plasma.

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An important advance in obtaining an alternate clinical source of FIB has been the cloning of cDNAs encoding the three different fibrinogen chains, and the publication of cDNA sequences. Rixon *et al.*,

5   *Biochemistry* 22: 3237 (1983); Chung *et al.*, *ibid* 3244; Chung *et al.*, *ibid* 3250. The structure of the FIB molecule is exceedingly complex. Each molecule of FIB consists of two sets of three different polypeptide chains, designated Aα, Bβ and Gγ, with molecular masses

10   of 66 kDa, 52 kDa and 46.5 kDa, respectively. The two half-molecules containing each set of chains are linked together by three disulfide bonds. In addition, a complex set of intra- and interchain disulfide bonds (there are a total of 29 disulfide bonds with no free sulfhydryl

15   groups) are involved in maintaining proper functional structure. Further, FIB is a glycoprotein with highly specific glycosylations. The molecule contains four carbohydrate chains, one each on the B, β, G and γ chains; the α and A chains contain no carbohydrate.

20   About 11 kDa of the total molecular mass of FIB (340 kDa) is attributable to this carbohydrate, added to the molecule post-translationally. In addition, isoforms of glycoproteins are known corresponding to differences in sialic acids on the carbohydrate chains. Proper

25   carbohydrate modification is required for functional activity of FIB.

These highly complex characteristics of the functional FIB molecule has made unpredictable and difficult the expression, assembly and secretion of fully

30   formed and functional recombinant molecules. Although the cDNA encoding the human FIB Aα chain has been expressed in bacteria (Lord, *DNA* 4:33 (1985)) this is of limited usefulness as the other fibrinogen chains that bear carbohydrates cannot be produced in prokaryotes.

35   Individual FIB chains have been expressed in COS1 (transformed monkey kidney fibroblast) cells. Danishevsky *et al.*, *Biochim. Biophys. Acta* 1048:202 (1990). In addition, transfecting COS1 cells with a combination of

cDNAs encoding the individual human fibrinogen subunit chains is reported to produce the holoprotein, but the amounts produced were small, and substantially less than the production achieved in the transgenic animal systems to be described below. Roy *et al.*, *J. Biol. Chem.*, 266:4758 (1991). The secretion of partially assembled or wholly unassembled and separate human FIB ("hFIB") or recombinant human FIB ("rhFIB") chains has not been reported for native or genetically engineered tissues. Chung *et al.* (1983), Danishevsky *et al* (1990). In addition, there are serious drawbacks to the use of mammalian cell tissue culture systems for production of FIB. These include the high costs of growth media, the labor intensive nature of such systems, and limited production capacity.

An important need persists for an efficient and relatively inexpensive means of producing clinically useful amounts of infectious particle-free rhFIB protein. The present invention satisfies this need. It has been surprisingly found that transgenic animals can be genetically engineered to produce and secrete into readily accessible body fluids therapeutically useful quantities of rhFIB. In addition to therapeutic uses involving replacement or addition therapy, the FIB of the invention finds us in a variety of applications, such as a "glue" in surgical procedures, as a delivery system for drugs, such as antibiotics or anti-parasitic agents, to wounds, as a food substitute, and for altering the composition of milk. These transgenic systems are described below.

SUMMARY OF THE INVENTION

In accordance with the present invention, there is provided a non-human mammalian animal that has stably integrated into its genome heterologous, *i.e.*, exogenously derived, polynucleotides that encode the $A\alpha$, $B\beta$ and $G\gamma$ polypeptide chains of human FIB, and that direct by means of regulatory and signal sequences the expression of biologically active rhFIB in mammary gland

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cells, such that newly synthesized fibrinogen is secreted into fluid compartments, particularly milk, of the animal. By integrating fewer than all of the three heterologous polynucleotides, individual chains of fibrinogen can be produced and may be secreted. By integrating heterologous polynucleotides that have been modified prior to administration to the host animal, modified FIB and products thereof can be produced.

It therefore is an object of the present invention to provide transgenic animals capable of producing rhFIB, polypeptide subunit chains of FIB, and FIB-derived proteins and protein products.

It is also an object of the invention to provide a means of producing rhFIB, polypeptide subunits thereof, and FIB-derived proteins and protein products in transgenic animals.

In a preferred embodiment of this aspect of the invention, lactating transgenic animals produce the rhFIB, FIB subunits and FIB-derived proteins in their mammary glands and secrete these products in their milk.

In another preferred embodiment, transgenic animals secrete the produced rhFIB, FIB subunits, and FIB-derived proteins in their blood and/or urine.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a sketch of the murine WAP gene.

Figure 2 is a sketch of the plasmid pUCWAP4 cassette vector containing ~ 2.6 kbp of WAP 5' promotor region, ~1.3 kbp of WAP 3' UTR and flanking 3' region.

Figure 3 is a sketch of the plasmid pUCWAP5 cassette vector containing the WAP fragment of pUCWAP4 with added Not I linkers.

Figure 4 is a sketch of the pUCWAP5 plasmid incorporating a polynucleotide encoding the FIB Aα 1 chain.

Figure 5 is a sketch of the pUCWAP5 plasmid incorporating a polynucleotide encoding the FIB Bβ 1 chain.

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Figure 6 is a sketch of the pUCWAP5 plasmid incorporating a polynucleotide encoding the FIB Gγ 1 chain.

Figure 7 shows the family tree of transgenic mice that have incorporated into their genome DNAs encoding FIB genes.

DETAILED DESCRIPTION OF THE INVENTION

Applicant has discovered a means of producing non-human transgenic mammalian animals that are genetically engineered to secrete into readily accessible body fluids such as milk, blood and urine recombinant human FIB ("rhFIB"), individual subunit chain polypeptides, and modified FIB subunit chains in amounts and in forms that are suitable for treating humans with genetic or acquired deficiencies of the normal protein.

The term "animal" here denotes all mammalian animals except humans. It also includes an individual animal in all stages of development, including embryonic and fetal stages. A "transgenic" animal is any animal containing cells that bear genetic information received, directly or indirectly, by deliberate genetic manipulation at the subcellular level, such as by microinjection or infection with recombinant virus.

"Transgenic" in the present context does not encompass classical crossbreeding or *in vitro* fertilization, but rather denotes animals in which one or more cells receive a recombinant DNA molecule. Although it is highly preferred that this molecule be integrated within the animal's chromosomes, the invention also contemplates the use of extrachromosomally replicating DNA sequences, such as might be engineered into yeast artificial chromosomes.

The term "germ cell line transgenic animal" refers to a transgenic animal in which the genetic information has been taken up and incorporated into a germ line cell, therefore conferring the ability to transfer the information to offspring. If such offspring, in fact,

possess some or all of that information, then they, too, are transgenic animals.

The information to be introduced into the animal is preferably foreign to the species of animal to which the recipient belongs (i.e., "heterologous"), but the information may also be foreign only to the particular individual recipient, or genetic information already possessed by the recipient. In the last case, the introduced gene may be differently expressed than is the native gene.

The transgenic animals of this invention may be any other than human, that produce milk, blood serum, and urine. Farm animals (pigs, goats, sheep, cows, horses, rabbits and the like), rodents (such as mice), and domestic pets (for example, cats and dogs) are included in the scope of this invention.

It is highly preferred that the transgenic animals of the present invention be produced by introducing into single cell embryos appropriate polynucleotides that encode human FIB, or subunit chain polypeptides or modified products thereof, in a manner such that these polynucleotides are stably integrated into the DNA of germ line cells of the mature animal and inherited in normal mendelian fashion.

Advances in technologies for embryo micromanipulation now permit introduction of heterologous DNA into fertilized mammalian ova. For instance, totipotent or pluripotent stem cells can be transformed by microinjection, calcium phosphate mediated precipitation, liposome fusion, retroviral infection or other means, the transformed cells are then introduced into the embryo, and the embryo then develops into a transgenic animal. In a preferred method, developing embryos are infected with a retrovirus containing the desired DNA, and transgenic animals produced from the infected embryo. In a most preferred method, however, the appropriate DNAs are coinjected into the pronucleus or cytoplasm of embryos, preferably at the single cell stage, and the embryos

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allowed to develop into mature transgenic animals. Those
techniques as well known. For instance, reviews of
standard laboratory procedures for microinjection of
heterologous DNAs into mammalian fertilized ova include:
5   Hogan *et al.*, *Manipulating the Mouse Embryo*, Cold Spring
Harbor Press, 1986; Krimpenfort *et al.*, *Bio/Technology* 9:
(1991); Palmiter *et al.*, *Cell*, 41:343 (1985); Kraemer *et
al.*, *Genetic Manipulation of the Early Mammalian Embryo*,
Cold Spring Harbor Laboratory Press, 1985; Hammer *et al.*,
10   *Nature*, 315:680 (1985); Wagner *et al.*, U.S. 5,175,385;
Krimpenfort *et al.*, U.S. 5,175,384, all of which are
incorporated by reference. The human FIB or FIB subunit
genes can be obtained by isolating them from an
appropriate genomic source (i.e., human liver which is
15   the natural organ for production of this protein) by
alternate methods which include preparation of cDNAs from
isolated mRNA templates, direct synthesis, or some
combination thereof. The cDNAs encoding individual FIB
chains can be fused, in proper reading frame, with
20   appropriate regulatory signals as described in detail
below, to produce a genetic construct that is then
amplified, for example, by preparation in a bacterial
vector, according to conventional methods (see, Sambrook
et al, *Molecular Cloning: A Laboratory Manual*, Cold
25   Spring Harbor Press, 1989 which is incorporated by
reference). The amplified construct is thereafter excised
from the vector and purified for use in producing
transgenic animals. Purification can be accomplished by
means of one or more cycles of anionic HPLC; alternate
30   techniques include ultracentrifugation through a sucrose
or NaCl gradient, gel electrolution followed by agarose
treatment and ethanol precipitation, or low pressure
chromatography. Purification by several cycles of HPLC
allows for remarkably high transformation frequencies, on
35   the order of 20% or more in both mice and pigs.

Although the present invention preferably entails the
use of DNA constructs that produce the desired or native
human FIB *per se*, the desired protein may also be

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produced as a fusion protein containing another protein. For example, the desired recombinant protein of this invention may be produced as part of a larger recombinant protein in order to stabilize the desired protein or to

5   make its purification from milk faster and easier. The fusion partners are then separated chemically or enzymatically, and the desired FIB isolated. Production of a fusion product between FIB and ß-lactoglobulin (BLG) is described in detail below.

10   The rhFIB may also be produced having the identical sequence as the native molecule, or it may be produced as a fragment or derivative. A variety of modified rhFIB or subunits thereof can be produced by altering a cloned DNA using the well-known techniques of *in vitro* mutagenesis

15   such as those set out in the references above.

Production of transgenic animals containing the gene for human FIB involves the use of cDNA or genomic DNA that encodes the α, ß, and γ chains of hFIB, Rixon *et al.*, 1983, above; Chung *et al.*, 1983, above. The full

20   length base sequence of each chain is provided in these references, which are incorporated by reference.

DNA constructs useful in the present invention provide a double stranded DNA sequence encoding FIB operably linked to all of the *cis*-acting signals

25   necessary for mammary gland-specific expression of this protein, post-translational glycosylation of two of the three sets of chains of FIB (Bß and Gγ), secretion of FIB into milk or other body fluids, and expression of full biological activity. As set out above, polynucleotides

30   encoding FIB or FIB subunit chains suitable for use in the invention can be isolated by standard techniques starting from the known polynucleotide sequences.

Modified FIB DNA sequences also can be employed in this invention. Useful modifications within this context

35   include, but are not limited to, those that alter post-translational modifications, size or active site of FIB, or that fuse this protein or portions thereof to another protein. Such modifications can be introduced into the

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protein by techniques well known in this art, such as by
synthesizing modified genes by ligation of overlapping
oligonucleotide or introducing mutations into the cloned
genes by, for example, oligonucleotide-mediated
5   mutagenesis.

The *cis*-acting regulatory regions useful in the
invention include the promoter that drives expression of
the FIB or FIB subunit chain genes. Highly preferred are
promoters that are specifically active in mammary gland
10   cells and that involve milk proteins. Among such
promoters, highly preferred are the whey acidic protein
(WAP), short and long α, β and kappa casein, α-
lactalbumin and β-lactoglobulin ("BLG") promoters.
Promoters may be selected on the basis of the protein
15   compositions of various milks.

For example, the WAP and BLG promoters are
particularly useful with transgenic rodents, pigs and
sheep. The rodent WAP short and long promoters have been
used to express the rat WAP gene, the human tPA gene and
20   the CD4 gene, while the sheep BLG promoter has been used
to express the sheep BLG gene, the human alpha-1-
antitrypsin gene and the human Factor IX gene. For
reviews, see Paleyanda *et al.*, *Recombinant Technology in
Hemostasis and Thrombosis*, eds. Hoyer *et al.*, Plenum
25   Press, NY 1991, page 197; Clark *et al.*, *TIBTECH*, 5:20
(1987). which are incorporated by reference. Preferred
among the promoters for carrying out the present
invention are the rodent casein and WAP promoters, and
the casein, α-lactalbumin and BLG promoters from porcine,
30   bovine, equine and ovine (pigs, sheep, goats, cows,
horses), rabbits, rodents and domestic pets (dogs and
cats). The genes for these promoters have been isolated
and their characterizations published; for reviews see
Clark *et al.* (1987), above, and Henninghausen, *Protein
35   Expression and Purification*, 1:3 (1990), which are
incorporated herein by reference.

The promoter may be isolated by carrying out
conventional restriction endonuclease and subcloning

steps. A mouse WAP promoter, isolated as a 2.6 kb EcoR1-Kpn1 fragment immediately 5' to the WAP signal sequence, is preferred, although the "long" WAP promoter (the 5' 4.2 kb Sau 3A-Kpn1 promoter of the mouse WAP gene, or a fragment thereof) is also suitable for carrying out this invention.

Important to the present invention are regulatory sequences that direct secretion of proteins into milk and/or other body fluids of the transgenic animal. In this regard, both homologous and heterologous regulatory sequences are useful in the invention. Generally, regulatory sequences known to direct the secretion of milk proteins, such as either signal peptides from milk or the nascent target polypeptide, can be used, although signal sequences can also be used in accordance with this invention that direct the secretion of expressed proteins into other body fluids, particularly blood and urine, such as signal sequences for coagulation proteins such as protein C and Factor VIII. Most preferred for the transgenic mouse is the regulatory sequences for the WAP protein.

Among the useful sequences that regulate transcription, in addition to the promoters discussed above, are enhancers, splice signals, transcription termination signals, and polyadenylation sites. Particularly useful in this regard are those that increase the efficiency of the transcription of the genes for fibrinogen in the mammary gland cells of the transgenic animals listed above. Preferred are transcription regulatory sequences for proteins highly expressed in the mammary gland cells (see above).

Preferably, the expression system or construct of this invention also includes a 3' untranslated region downstream of the DNA sequence encoding the desired recombinant protein, or the milk protein gene used for regulation. This region apparently stabilizes the RNA transcript of the expression system and thus increases the yield of the desired protein. Among the 3'

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untranslated regions useful in this regard are sequences
that provide a poly A signal. Such sequences may be
derived, e.g., from the SV 40 small t antigen, the casein
3' untranslated region, or other 3' untranslated
5   sequences well known in this art. Preferably, the 3'
untranslated region is derived from a milk-specific
protein. The stabilizing effect of this region's poly A
transcript is important in stabilizing the mRNA of the
expression sequence. Also important in increasing the
10   efficiency of expression of FIB are ribosome binding
sites.   Likewise, sequences that regulate the post-
translational modification of FIB subunits are useful in
the invention.

Thus, in accordance with this invention, a
15   double-stranded chimeric DNA including genes encoding the
FIB subunit chains operably linked to cis-acting
regulatory sequences that promote efficient expression of
the former in milk and/or other body fluids are
introduced into an embryo where they are integrated into
20   the embryonic genome and become part of the inheritable
genetic endowment of all of the animals cells, including
the germ line cells, of the adult that matures from the
embryo. The FIB thus expressed and secreted into milk is
immunologically reactive, as measured by an ELISA assay
25   as described below.

Where the synthesis of a FIB subunit chain may be
limiting in the production of the holoprotein, expression
of this chain an be increased by placing the gene in a
different genomic locus. This other locus can contain a
30   DNA sequence under the same or a different regulatory
sequence than the other two FIB sequences.

In a particularly preferred embodiment the transgenes
of the invention generally consist of milk protein
upstream and downstream and flanking sequences in
35   combination, separately, with each of the three
translated portions of the cDNA sequences representing
the three FIB subunit chains. A native 5'-WAP regulatory
sequence ending in an accessible restriction site

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immediately before/at the ATG codon may be ligated to each of the respective restriction sites which occur at the ATG of translatable sequences with no linker sequences derived from the chains of human FIB. A
5   restriction site may be made through site mutagenesis of three bases located in each 3'-untranslatable sequences of the three FIB cDNAs. This preserves the stop sequence while adding a restriction site immediately after each of the translatable sequences of the FIB cDNAs. Each of the
10   combined 5'-regulatory and FIB translatable sequences ending in a particular restriction site may then be ligated to a corresponding restriction site which occurs at the beginning of the 3'-untranslated region of WAP and adjoining WAP 3'-flanking region. This construction motif
15   enables native 5'-regulatory and 3'-UTR of the milk protein genes to be immediately juxtaposed without intervening sequences (but see above relating to the insertion of the Bß gene at a different locus). Particular restriction sites at the ends of all
20   constructs may be selected in order to facilitate concatenation of Aα, Bß and Gγ constructs into a single domain within the animal genome.

Although the above general descriptions of the DNA constructs of the invention have been given in terms of
25   the WAP promoter, it is emphasized that other suitable promoters (see above for discussion) may be ligated to the fibrinogen encoding polynucleotides in a similar manner. By way of illustration, the following discussion describes the use of the bovine lactoglobulin ("BLG")
30   promoter to increase the efficiency of expression of FIB and FIB-BLG fusion proteins in mammary glands.

Using techniques described above, fibrinogen encoding cDNA may be inserted into an ovine BLG gene. For instance, in order to produce such a construction, the
35   11.2 Kbp ovine BLG gene may be modified to possess a unique EcoRV site upstream of the initiator ATG codon in the vector pUCXSRV. The sequence around this region is changed as follows:

```
                                    PvuI          MetLys
        Seq. ID No. 1 Natural    CCCCAGCTGCAGCCATGAAG

                                    EcoRV                 MetLys
        Seq. ID No. 2 pUCXSRV   CCCCAGGGATATCCCTGCAGCCATGAAG
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5   This allows the cloning of blunt end fragments
upstream of the BLG gene. The 1.5 kbp fragment from a
plasmid (e.g., pWAP FIB) containing a cDNA encoding human
fibrinogen is isolated, blunt ends are generated with T4
DNA polymer, and the product is ligated to EcoRV-cleaved
10   pUCXSRV. Following transformation of E. coli with this
plasmid, clones that are produced can be characterized by
restriction analysis of plasmid DNA prepared by a
mini-prep method and by determination of the nucleotide
sequence around the 5' and 3' cloning junctions for the
15   DNA. Clones having the desired structure can be used to
produce transgenic rodents, pigs, sheep, cows, horses and
other farm animals and domestic pets (cats and dogs) that
secrete a FIB-BLG fusion product into their biological
fluids as described below.
20   A human fibrinogen genomic sequence also may be fused
to the ovine BLG promoter illustrated in the following
discussion. DNA sequences encoding ovine BLG in plasmid
pUCXSRV are deleted to generate a vector containing only
ovine BLG promoter sequences(pUCSV). As with pUCSRV,
25   blunt ended fragments may be fused to this promoter
region by ligation to a unique EcoRV site. The sequences
5' to this site are identical in both plasmids.
        Genomic FIB sequences of greater than about 15 kbp
can be introduced into transgenic animals, despite their
30   length, through the use of cosmids with overlapping FIB
sequences, whereupon the necessary assembly of an entire
genomic polynucleotide encoding hFIB is achieved by
homologous recombination in vivo after microinjection
into an embryo cell. In constructs useful in the
35   foregoing example, a plasmid in which the FIB genomic
sequences are fused to ovine BLG 3' flanking sequences
just after the fibrinogen translation termination codon

to ensure proper transcription, termination and polyadenylation. The hFIB gene fused to ovine BLG 3' flanking sequences is excised from the plasmid, the 3' overhangs repaired using Klenow enzyme, and the product ligated to EcoRV-cleaved pUCSR. Following transformation of *E. coli*, the resulting clones are characterized by restriction DNA analysis and by determining the nucleotide sequences around the 5' and 3' cloning junctions. Clones having the desired structure may be introduced into fertilized animal ova for production of transgenic animals.

A variety of vectors based on the BLG gene may be constructed. In constructs based on this approach, the sequences encoding the ovine BLG protein are deleted, but the 5' promoter sequences are retained. Each may possess a different complement of introns from the ovine gene and a unique EcoRV site allowing the cloning of blunt ended fragments between the promoter and 3' flanking region of the gene. However, each contains the BLG promoter, the EcoRV site and the BLG 3'-flanking sequence. For each recombinant, the 1.5 kbp KpnI fragment from pWAP FIB is isolated, blunt ends generated as above, and the product ligated to EcoRV-cleaved vector sequences. Those constructs with the proper structures (determined as described above) may be used to express fibrinogen in the biological fluids of transgenic animals.

We have observed that doubly-transgenic mice produced having native BLG genomic sequences which are injected as separate constructs to be concatenated *in vivo* as additional flanking sequences to the BLG target cDNA construct (such as, BLG promoter-Intron I-EcoRV-Intron VI-BLG 3' flank plus BLG) give higher expression more frequently than that observed with the use of constructs of the BLG promoter-FIB cDNA-BLG gene or BLG promoter-FIB genomic (± BLG 3' end). Intact or native BLG genomic sequences that are preligated to the BLG-cDNA target may give the same advantage. This same principle can be extended to WAP genomic sequences.

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   Obtaining milk from a transgenic animal according to
the present invention is accomplished by conventional
means. See, e.g., McBurney et al., J. Lab. Clin. Med.
64:485 (1964); Velander et al., Proc Natl. Acad. Sci.,
5   USA 89:12003 (1992), which are incorporated by reference.
Fibrinogen or subunit chains or protein products thereof
can be isolated and purified from milk or urine by
conventional means without deleteriously affecting
activity. A highly preferred method consists of a
10 combination of anion exchange and immunochromatographies,
cryoprecipitations, zinc ion-induced precipitation of
either whole milk or milk whey (defatted milk) proteins.
For these techniques, see Bringe et al., J. Dairy Res.
56:543 (1989) which is incorporated herein by reference.
15   Milk is known to contain a number of proteases that
have the potential to degrade foreign proteins. These
include in the main an alkaline protease with tryptic and
chymotryptic activities, a serine protease, a
chymotrypsin-like enzyme, an aminopeptidase and an acid
20 protease. Clark et al. (1987) above. It may be desirable
therefore to protect newly secreted FIB or subunit
chains thereof against proteolytic degradation. Such
precautions include rapid processing of the milk after
collection and addition to the milk of well known
25 inhibitors of proteolysis, such as are listed in Sigma
Chemical Co. Catalog, 1993 edition, page 850,
incorporated herein by reference.
   We have used two sandwich ELISA formats to detect and
quantify FIB in milk whey. One technique is a polyclonal
30 antibody capture/FIB/monoclonal antibody detection system
("P/M") and the other which uses polyclonal antibodies
both for capture and for detection ("P/P"). Both methods
are described below. The P/M system benefits from
greater specificity for hFIB as it recognizes only one
35 epitope of about 6 to 20 amino acids in length specific
to hFIB. However, because the P/M system recognizes only
a single epitope, detection sensitivity is reduced. In
contrast, the P/P system will provide less specificity as

it recognizes many epitopes, but at the same time will
provide greater detection sensitivity. As these multiple
epitopes include epitopes conserved between mouse and
human FIB, the background "FIB" signal is increased when
5   measuring rhFIB in mouse milk by the P/P system.
Detailed sandwich ELISA procedures are provided in
EXAMPLE 7 below.

The present invention is further described by
reference to the following illustrative examples.

10   EXAMPLE 1
        WAP-Fibrinogen cDNA Constructs For Expressing
              Fibrinogen In Transgenic Animals
Construction of cassette vectors
        Fibrinogen subunit chain DNAs, tissue-specific
15 promoters, and secretion signals were obtained from
sources described above. Fibrinogen subunit chain cDNAs
were cloned into a modified pUC 18 vector, and grown up
in *E. coli* JM109.
        A pUC18 vector (GIBCO-BRL, Gaithersburg, MD) was
20 digested with HindIII + EcoRI restriction endonucleases,
blunted with T4 DNA polymerase in the presence of 100 mM
dNTPs, and a Not I linker was ligated into the former
HindIII-EcoRI multiple cloning site. This modified pUC
fragment was additionally digested with Not I+ enzyme to
25 remove extra (multiple copies) NotI linker sequences
arising from ligation, and then religated and grown up in
E. coli JM 109. This procedure modified the pUC18 vector
by removing the entire multiple cloning region of pUC18
(including the Kpn I site) and replacing it with a Not I
30 restriction site. The new vector was designated
pUCNot1+.
        A pUC vector containing ~2.6 kbp of WAP 5' promoter
region, ~1.3 kbp of WAP 3' UTR and flanking 3', but no
WAP coding or intronic regions was constructed
35 (designated cassette vector pUCWAP4, Figure 2; see WAP
gene, Figure 1, and cassette vector pUCWAP5, Figure 3).
The entire murine WAP gene including 2.6 kb of 5'

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untranslated sequence and 3' untranslated region may be
obtained from Dr. I. Hennighausen, National Institutes of
Health, Bethesda, Maryland.   See, Campbell *et al.*,
Nucleic Acids Res., 12:8685 (1984). The WAP fragment
5   contained within the WAP4 vector contains a ~2.6 kbp WAP
promoter 5' region beginning at EcoRI#1 and ending at the
translational start site of WAP which is immediately
downstream of the unique Kpn I endonuclease restriction
site (see Figure 1).   To this KpnI site was ligated a
10   fragment of ~1.3 kbp of WAP UTR and flanking 3' sequence.
This WAP 3' DNA included the region from immediately
downstream of the WAP stop codon down to the EcoRI#2
site.   The WAP fragment contained in WAP4 was excised
from the pUC vector using EcoRI, and then blunted, and
15   NotI linkers were added, further trimmed by NotI
digestion, and ligated into the pUCNotI+ plasmid which
had been linearized with NotI restriction endonuclease.
The resulting plasmid was designated pUCWAP5 (see Figure
3).

20   Amplification by PCR of fibrinogen subunit chain cDNAs
        Each of the cDNAs for Aα, Bβ, and Gγ chains for human
fibrinogen were individually modified and amplified by
polymerase chain reaction (PCR) to create KpnI
endonuclease restriction sites on their 5' and 3' ends.
25   The 5' KpnI site was engineered by PCR using the primers
[containing the 6 base sequence (GGTACC shown underlined
in Table 1)] that immediately flanks the ATG start codon
in the cDNAs of Aα, Bβ, and Gγ chains for hFIB.   After
amplification, the ends of the extension using PCR
30   products were blunted by T4-polymerase (in the presence
of deoxynucleosidetriphosphates to inhibit processive
exonuclease activity.   In a similar fashion, a KpnI site
was engineered by site modification into the 6 base
sequence GGTACC shown underlined in Table 1 immediately
35   flanking the stop sequence in the 3' UTR of each cDNA for
Aα, Bβ, and Gγ chains for hFIB.   The complement of the
stop sequences is shown in bold in the 3' primers in
Table 1.

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Table 1

Oligonucleotides for Amplifying FIB Subunit Chain cDNA

Seq. ID No. 3

     Aα 5' GCTA<u>GGTACC</u>ATGTTTTCCATGAGGATCGT

5   Seq. ID No. 4

     Aα 3' CAGT<u>GGTACC</u>CTAGACAGGGCGAGATTTAG

Seq. ID No. 5

     Bβ 5' GCTA<u>GGTACC</u>ATGAAAAGAATGGTTTCGTG

Seq. ID No. 6

10   Bβ 3' CAGT<u>GGTACC</u>CTATTGCTGTGGGAAGAAGG

Seq. ID No. 7

     Gγ 5' GCTA<u>GGTACC</u>ATGAGTTGGTCCTTGCACCC

Seq. ID No. 8

     Gγ 3' CAGT<u>GGTACC</u>TTAAACGTCTCCAGCCTGTT

15   GGTACC = KpnI site

      ATG = start codon

      CTA and TTA = stop codon

    <u>Construction of pUC plasmids containing</u>

       <u>fibrinogen subunit chain cDNA</u>

20   The blunted PCR products of the cDNAs for the Bβ and Gγ chains of human fibrinogen were digested with <u>KpnI</u> restriction endonuclease. In the case of the Aα chain, the PCR product was blunt-end cloned into pUCNotI+ (which had been digested with <u>NotI</u> and blunted with T4 Polymerase) prior to partial <u>KpnI</u> digestion. This intermediate cloning and partial digestion step was necessary to generate intact coding fragment due to the presence of an internal <u>KpnI</u> site within the Aα chain cDNA. The intact Aα chain cDNA fragment was selected by gel electrophoresis, and cloned into the <u>KpnI</u> site at the junction between the WAP 5' promoter and WAP 3' UTR/flanking sequences within the pUCWAP5 plasmid. The <u>KpnI</u>-digested PCR products from Bβ and Gγ chains for human fibrinogen were each directly cloned into a pUCWAP5 plasmid at the <u>KpnI</u> site. Separate electroporation transformation reactions were done on *E.coli* using each of the three pUCWAP5/fibrinogen cDNA preparations, and

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colonies were picked and grown up in TB ampicillin broth.
Plasmid preparations from these colonies were analyzed by
restriction enzyme digestion and gel electrophoresis.
The correct size and orientations were selected and one
5   clone for each WAP-fibrinogen cDNA construct was
sequenced at the WAP promoter 5':fibrinogen cDNA and
fibrinogen cDNA:WAP 3' UTR and flanking junctions.
Schematics summarizing the construction of the WAP-Aα
(about 5.8 kbp), -Bβ (about 5.4 kbp), and -Gγ (about 5.2
10   kbp) cDNA plasmid and linearized transgenes for human
fibrinogen are given in Figures 4, 5, and 6,
respectively.

EXAMPLE 2
15   **Preparation of DNAs for Microinjection**
     The intact and linearized WAP 5' promoter/fibrinogen
cDNA/WAP 3' UTR and flanking fragments were excised from
each pUCWAP5 plasmids by Not_I restriction endonuclease
digestion and purified by low melting point agarose
20   electrophoresis. The DNA:agarose band was cut from the
gel slab. The agarose band was then treated with agarase
to degrade and remove agarose contamination
     After digestion, the solution containing the cDNA was
brought to 10 mM Mg2+, 20 mM EDTA and 0.1% SDS and then
25   extracted with phenol/chloroform. DNA was precipitated
from the aqueous layer with 2.5 volumes of ethanol in the
presence of 0.3 M sodium acetate at -20° C overnight.
After centrifugation, the pellet was washed with 70%
ethanol, dried, and each of the constructs was
30   resuspended and dissolved in Brinster microinjection
buffer to a total (α, β, plus γ) concentration of about
1.2 to 5 μg/ml. Brinster *et al.*, *Proc Natl. Acad. Sci.
USA* 82:4438 (1985), incorporated by reference.
                         EXAMPLE 3
35   **Transgenic Mice**
     Transgenic mice were produced essentially as
described by Hogan *et al.*, *Manipulating The Mouse Embryo*,

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Cold Spring Harbor Press, 1986, incorporated by
reference. The procedures employed are outlined below.

Glass needles for micro-injection were prepared using
a micropipet puller and microforge. Injections were
5   performed using a Nikon microscope having Hoffman
Modulation Contrast optics, with Narashigi
micromanipulators and a pico-injector driven by N_2
(Narashigi).

Fertilized mouse embryos were surgically removed from
10 the oviducts of superovulated female CD-1 mice and placed
into M2 medium. Cumulus cells were removed from the
embryos with hyaluronidase at 300 μg/ml. The embryos
were then rinsed in new M2 medium, and transferred into
M16 medium for storage at 37° C prior to injection.

15 Stock solutions containing about 1.2 μg/ml (having
about 0.4 μg/ml each of the linearized constructs
containing the Aα, Bβ, and Gγ chains cDNAs for human
fibrinogen, or about 60-70 copies/pl of each construct)
were prepared and microinjected into the pronuclei of 1
20 cell mouse embryos. In addition, stock solutions
containing about 5 μg/ml total DNA (having about 1.7
μg/ml of linearized constructs containing each of the Aα,
Bβ, and Gγ chains cDNAs for human fibrinogen or about 200
copies/pl of each construct) were prepared and
25 microinjected into the pronuclei of 1 cell mouse embryos.

After injecting the DNA solution into the male
pronucleus, embryos were implanted into avertin-
anesthetized CD-1 recipient females made pseudo-pregnant
by mating with vasectomized males. About 25-30
30 microinjected mouse embryos per recipient were
transferred into pseudopregnant females. Embryos were
allowed to come to term and the newborn mice were
analyzed for the presence of the transgene by PCR using
murine WAP and a FIB-specific primer as described below.

35 Table 2 summarizes the embryo transfer data from an
experiment that produced transgenic fibrinogen-producing
mice. Separate PCR analysis for detection of each of the
cDNAs of fibrinogen was done on DNA purified from

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biopsied tail tissues by the method shown in Example 4
below. The oligonucleotide primers shown in Table 3 were
used to detect each of the FIB cDNAs. Detection of each
chain required a WAP oligonucleotide (no. 1 in Table 3)
5   and one of the other oligonucleotides (Nos. 2, 3 or 4).

Table 2

Transfers of Fibrinogen Constructs

Thirty transfers with the FIB constructs were
performed. The three subunit chain constructs were co-
10 injected at total DNA concentrations of 1.2 μg/ml and 5
μg/ml or 200 total copies/pl and 800 total copies/pl,
respectively. Fourteen transfers were made with 1.2
μg/ml-injected embryos and sixteen with 5 μg/ml-inject
embryos. Eighteen Founder ("Fo") animals were produced
15 that carried at least one construct. Eight Fo mice were
mated with control mice which resulted in five lines
transmitting the transgene. First generation offspring
("F1") from lines 1,23 and 85 have been bred and litters
produced.

20   1.2μg/ml   5μg/ml

Transfers:	14	16
Pups alive:	68	69
Embryos injected:	696	1098
Embryos transferred:	480	675
Embryos per transferred:	34.2	42.1
Pups per litter	6.2	5.3
Transfers pregnant:	11	13
Pregnancy rate:	78%	81%
Mice tested:	68	69
Number transgenic:	7	11
Percent transgenic:	10%	15%
% Positive for α,β,γ:	85%	72%
α,β,γ Transgenic females:	3	3

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Table 3

Oligonucleotide Primers Used for PCR Detection of Human Fibrinogen Transgenic Mouse

Seq. ID No. 9. Sense strand. WAP-specific oligonucleotides.

            5'-CTGTGTGGCCAAGAAGGAAGTGTTG-3'

Seq. ID No. 10. Antisense oligonucleotide. Aα chain detection.

            5'-GATGTCTTTCCACAACCCTTGGGC-3'

Seq. ID No. 11. Antisense oligonucleotide. Bβ chain detection.

            5'-CCCGATAGCCACCTCCTCTGATG-3'

Seq. ID No. 12. Antisense oligonucleotide. Gγ chain detection.

            5'-CCTGGACTTCAAAGTAGCAGCGTC-3'

A summary of the types of fibrinogen transgenic mice detected in founder and offspring of founder mice is given in Table 4. A listing of the positive FIB triple construct founders (Fo) and a listing of β-chain positive transgenic mice are also given in Table 4.

Table 4

Transmission of Fibrinogen Transgenes

Mouse No.	Transmission Frequency	Genes Transmitted
1 (α,β,γ)	9/13 (69%)	α,β,γ
23 (α,β,γ)	6/12 (50%)	α,β,γ (3/6); β only 3/6)
109 (α,β,γ)	5/11 (45%)	α,β,γ
112 (α,β,γ)	5/8 (62%)	α,β,γ (4/6) α only (1/6) α,γ only (1/6)
85 (α,β)	1/6 (16%)	β only
113 (αβγ)	0/8	None
89 (α)	0/6	None

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Positive Fibrinogen Triple Construct F1's

1-2,1-5,1-6,1-11,1-12,1-14,1-16,1-17,1-20,23-3,23-4,23-

12,109-3,109-4,109-5,109-10,109-11,112-3,112-6,112-7,112-

8

β-Chain Positive Mice

23-2,23-7,23-11,85-2

All embryos were injected with the three FIB constructs at a total DNA concentration of 5 μg/ml or about 200-300 copies of each construct per pl.

A family tree of transgene passage from F0 to F1 females is shown Figure 7. Males were not retained.

EXAMPLE 4

Transgenic Animal DNA

DNA can be prepared from tissue of a transgenic animal of any species by the method exemplified below for mice. Marmur, *J. Mol. Biol.* 3:208 (1961), incorporated by reference.

A 5 mm piece of mouse tail was removed from young, potentially transgenic mice at weaning (3 weeks) age, and frozen in liquid nitrogen. To the frozen tissue was added 840 μl of Lysing Solution (8 mM EDTA-0.8% SDS-0.8% 2-mercaptoethanol-80 μg/ml Proteinase K-1 M sodium chlorate in 40 mM TRIS buffer pH 8.0 and 120 mM NaCl, and the mixture incubated at 50°C. The mixture was then extracted with 250 μl of phenol/chloroform/isoamyl alcohol (25:24:1) for 10-15 seconds, then centrifuged for 10 minutes. The supernatant fluid (about 830 μl) was removed to a fresh tube, and a DNA clot produced by vortexing the solution with 0.6 vols. of isopropanol. The mother liquor was decanted, and the DNA clot rinsed twice with 80% ethanol. The DNA clot was isolated by 5 mins. of centrifugation, aspiration of the supernatant fluid, and air drying of the clot with a stream of air for 10 mins.

The DNA clot was dissolved in 250 μl of TE buffer (10 mM Tris.HCl, pH 7.0-1mM EDTA, and the solution treated with 10 μl of a RNase mixture (1 mg/ml RNAse A and 4,000 units/ml RNAse T1) for 1 h at 37°C. This mixture was shaken with 50 μl of a 24:1 (v/v) solution of

chloroform-isoamyl alcohol for 5-10 secs., centrifuged, and the supernatant fluid transferred to a fresh tube.

The recovered supernatant fluid above was mixed sequentially with 25 µl of 3 M sodium acetate and 0.5 ml
5   of 95% ethanol. The supernatant fluid was decanted from the precipitated DNA, and the precipitate washed with 80% ethanol. The purified DNA was isolated by centrifugation, air dried, then dissolved in 150 µl of TE.

Essentially the same technique was used to prepare
10 DNA from pigs, and the same or similar techniques can be used to prepare DNA from other animals.

PCR reactions on DNA samples isolated from transgenic animal tissue samples were carried out conventionally, as described above. Concentrations of DNA
15 isolates were determined by measuring the absorbancy at 260 nm of 1:20 dilutions of the TE solutions described above. A portion of the sample was then diluted into 100 µl of TE to a concentration of 30 ng/µl.

PCR reaction mixtures (20 µl volume)

Component	Volume (µl)	Concentration
20	HOH	13.25	-
10X Taq buffer	2.5	1
dNTPs	2.0	0.2 mM
MgCl$_2$	1.5	1.5 mM
25	Primer 1	0.3125	0.5 µM
Primer 2	0.3125	0.5 µM
Taq polymerase	0.125	25 µunits/µl
DNA template	1.0	1.2 ng/µl

Procedure

30   Embryo lysing buffer (5 µl, 0.9% Nonidet P-40 and 0.9% Tween 20 in 20 mM TRIS buffer) was added to each PCR tube along with 1 µl of prepared DNA solution, and the mixture overlaid with 25 µl of mineral oil. The tubes were placed in an automatic temperature cycler (such as
35 that manufactured by N.J. Research) and heated to 98°C for 2 mins.; the temperature was then brought down to 85°C.

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PCR reaction mixture (20 μl) was added to each tube and
40 cycles of the following protocol carried out:
      Denaturation at 96°C for 20 seconds
      Annealing at 55°C for 1 minute
5   Elongation at 75°C for 30 seconds
      PCR reaction products were analyzed by running 20%
of the reaction mixture products on agarose gels and
identifying fragment sizes by comparison with marker DNA
fragments.
10   Figure 7 shows that a variety of founder animals
having A+ and/or B+ and/or G+ constructs can transmit all
three or some of the A+, B+, and G+ transgenes. All
animals were produced from embryos having coinjections of
the same DNA solution containing all of the A+, B+, and
15   G+ constructs. Detection of A+, B+ and G+ DNAs was done
by PCR as described above. Founder animals 1, 23, 109 and
112 had all three transgenes. Founder animal 85 had only
A+ and B+. These founder animals were outbred with
control (nontransgenic) mice. Animals 1 and 109 passed
20   all three transgenes to 5 of 13 and 5 of 11 offspring,
respectively, which is typical of Mendelian genomic
transmission for a single allele. Founder animals 23 and
112 transmitted some transgene to 6 of 12 and 7 of 8
animals, respectively. Several offspring of animal 23 had
25   only B+, while one offspring of animal 112 had only A+
and another had A+ and G+. Thus, founder animals 23 and
112 are examples of triple transgenic animals having a
confirmed separate B+ allele in the case of animal 23 and
having a separate A+ and B+ allele in the case of animal
30   112. Founder animal 85 is also an example of a mouse with
a B+ only allele. Thus, crossbreeding of animals having
separate alleles and therefore loci for each gene can be
done in order to increase expression (the control over a
rate limited B+ chain may be overcome by having a B+
35   allele with a more inducible loci). Furthermore, double
and triple heterozygotes of the same gene (either A+
and/or B+ and/or G+) can be achieved to boost production
levels (i.e., produce a A+ B+ G+, B+ animal by

crossbreeding a B+ animal, and further crossbreeding that A+B+G+,B+ animal with a different B+ line to obtain A+ B+ G+,B+ B+).

EXAMPLE 5
Transgenic Pigs

Embryos were recovered from the oviduct. They were placed into a 1.5 ml microfuge tube containing approximately 0.5 ml embryo transfer media (phosphate buffered saline + 10% fetal calf serum, Gibco BRL). These were then centrifuged for 12 minutes at 16,000 x g RCF (13,450 RPM) in a microcentrifuge (Allied Instruments, model 235C). Embryos were removed from the microfuge tube with a drawn and polished Pasteur pipette and placed into a 35 mm petri dish for examination. If the cytoplasm was still opaque with lipid such that pronuclei were not visible, the embryos were centrifuged again for 15 minutes. Embryos to be microinjected were placed into a microdrop of media (approximately 100 μl) in the center of the lid of a 100 mm petri dish. Silicone oil was used to cover the microdrop and fill the lid to prevent media from evaporating. The petri dish lid containing the embryos was set onto an inverted microscope (Carl Zeiss) equipped with both a heated stage and Hoffman Modulation Contrast optics (200 x final magnification). A finely drawn (Kopf Vertical Pipette Puller, model 720) and polished (Narishige microforge, model MF-35) micropipette was used to stabilize the embryos while about 1 - 2 picoliters of HPLC-purified DNA solution containing approximately 200-500 copies of DNA construct was delivered into the male pronucleus with another finely drawn micropipette. Embryos surviving the microinjection process as judged by morphological observation were loaded into a polypropylene tube (2mm ID) for transfer into the recipient pig. Seven surgical transfers of pig embryos were made. These embryos had been coinjected with the WAP-fibrinogen cDNA constructs used in the above mentioned transgenic fibrinogen mice Only one of these recipient gilts was confirmed pregnant

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by ultrasound. The (apparent) non-pregnant animals were
kept to tentative term as their (potential) litter sizes
may have been too small to detect by ultrasound analysis
(In the past, we have had 3 litters of 4 piglets or less
which were not detected by ultrasound. Some of these
litters produced transgenic animals.)

Table 5
Fibrinogen Porcine Embryo Data

	Expt. 1	Expt. 2			
			Transfer Totals 10/25/93		ID
Number of donors:	8	8	Transfer#1 50	embryos	#129
Number of ovulations:	261	283	Transfer#2 47	embryos	#147
Embryos collected:	250	296	Transfer#3 44	embryos	#108
Number of UFO's:	99	77			
UFO's that cleaved:	29	6			
Number injected:	141	219	10/28		
Number transferred:	141	200	Transfer#1 50	embryos	#114
Transfers:	3	4	Transfer#2 50	embryos	#139
Embryos/Transfer:	47	50	Transfer#3 50	embryos	#140
Ovulations/Pig:	32.6	35.3	Transfer#4 50	embryos	#131
Embryos/Pig:	31.2	37			

All embryos were injected with the three fibrinogen
constructs at a total DNA concentration of 5 μg/ml or
about 200-300 copies of each construct per pl.

EXAMPLE 6

Preparation of Milk and Whey from Transgenic Animals

Lactating mice were milked once on about day 12 of lactation. The mice were first separated from their young for approximately 5 hours. They were then anesthetized with 0.4 ml avertin at 2.5% (im.), and 0.2 ml oxytocin was then administered at 2.5 IU/ml (ip.) to permit release of the milk. A milking device consisting of a vacuum pump (2.5 psi) and syringe with an Eppendorf tip was used to direct milk into an eppendorf tube. As noted above, milk should not be placed on ice because of cryoprecipitation of fibrinogen.

To prepare whey, milk was diluted 1:1 with TRIS buffer (0.03 M Tris pH 7.4; 0.06 M NaCl) and centrifuged in a TLA-100 rotor in a Beckman TL-100 table top ultracentrifuge at 51,000 rpm (89,000 x g) for 30 minutes at room temperature. After centrifugation, the whey (infranatant fluid) was collected with an 18 gauge needle, leaving the casein pellet and upper cream layer in the tube. To remove solids or cream that co-transferred during the initial recovery, the whey obtained from the first centrifugation was subjected to a second spin at 12,000 rpm for 30 mins. in a TMA-4 rotor in a Tomy MTX-150 centrifuge. Following the second spin, the whey was recovered as before.

EXAMPLE 7

Determination of Fibrinogen in Milk by a Sandwich ELISA

A sandwich ELISA was used to measure the amount of FIB protein produced by founder and progenic transgenic animals in the diluted skim or defatted milk (here termed "whey"); the whey value is used to estimate the concentration of FIB in whole milk.

Generally, in the first step of the P/M ELISA system, a fibrinogen or fibrinogen chain analyte-containing sample (for example, whey) is incubated with a mouse anti-human fibrinogen mAb (clonal number 311, American Diagnostics) that is reactive with the alpha

chain of fibrinogen to form a mAb-fibrinogen (or chain) first complex. This first complex is then "captured" by incubation with rabbit polyclonal anti-human fibrinogen antibodies immobilized in microliter plate wells. In the detection step, the captured fibrinogen (or alpha chains) is contacted with labeled (horse radish peroxidase) anti-mouse IgG, and the amount of label quantified by reaction with o-phenylenediamine and measurement of absorbency at 490 nm; this is a measure of the amount of analyte present. Standard curves were obtained by spiking the whey of control (nontransgenic) animals with pure reference human FIB material. Thus, the presence of background amounts of FIB is normalized by the standard curve. This sandwich ELISA gives very low background values (about 0.06 absorbancy units at 490 nm) for mouse whey alone with no human FIB. Note the typical signals given in Table 6 are about 0.25 or higher absorbancy units at this wave length.

In the P/P system, Immulon II 96-well microliter plates were coated with 100 μl/well of 1:500 diluted rabbit anti-human FIB (Celsus cat. no. 60960) in 0.1 M $NaHCO_3$-0.1 M NaCl, pH 9.6, for 24 h at refrigerator temperatures. Coated wells were washed three times with Wash Buffer (25 mM Tris-HCl-50 mM NaCl-0.5% Tween-20, pH 7.0) and blocked for 20 mins. with Dilution Buffer (1 mg/ml PEG in 25 mM Tris-HCl-50 mM NaCl, pH 7.0) at room temperature. 100 μl of standard human FIB (100 ng/ml to 1.9 ng/ml range) and/or 100 μl of diluted milk sample were added to each well, and the mixtures incubated at 37°C for 45 mins. The wells were washed and blocked as above for 10 mins. at room temperature. 100 μl of 1:1000 diluted horse radish peroxide-conjugated rabbit anti-human FIB were added to each well, and the mixtures incubated at 37°C for 45 mins. After washing the wells as above, 100 μl of o-phenylenediamine solution (1 tablet/5 ml diluent) were added to each well. After waiting about 2-3 mins. for color development, reactions were stopped

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by the addition to each well of 100 μl of 3 N H^{2}SO$_4$.
Plates were then read at 490 nm.

Table 6 presents the results of the analyses of
rhFIB by the P/M ELISA system described above in the
5   first lactation whey (and in whole milk by calculation)
of three founder (Fo) transgenic mice whose genome
contained each of the Aα, Bβ, and Gγ chains cDNAs for
human fibrinogen. In this experiment, "whey" refers to
skim milk diluted 1.7X with EDTA to solubilize the
10   caseins. The averages of four dilutions of each whey
sample for the three test animals was about 16.3, 3.86
and 7.55 μg/ml, respectively. The corresponding values
for whole milk were 27.6, 6.56 and 12.8. Second lactation
production of rhFIB was similar.

15   In another experiment with the same animals the
first lactation whey fibrinogen concentrations averaged
about 22 $\pm$ 4 (animal 1), 9 $\pm$ 2 (animal 22) and 11 $\pm$ 3
(animal 23) μg/ml. The second lactation of animal 1
produced 17 $\pm$ 3 μg/ml whey.

20   The P/M ELISA system was used to assay for rhFIB in
the third lactation milk of mouse no. 1/Fo and the first
lactation of mouse no. 1 female offspring (1-11/F1). As
shown in the data of Table 7, mouse no. 1/Fo had about 71
μg/ml, and her offspring (no. 1-11/F1) had about 53 μg/ml
25   total FIB signal. Thus, the amount of rFIB after
subtracting background is calculated to be about 50 μg/ml
for mouse no. 1/Fo and about 32 μg/ml for offspring 1-
11/F1, respectively, over that of endogenous FIB present
from serum to milk passover. The slightly lower value (28
30   μg/ml) of the P/M assay of the previous lactation of
mouse no. 1/Fo is not significantly different than the
P/P value when lower detection sensitivity of the former
method is taken into account. It is noted that collection
of mouse milk is inherently traumatic and leads to serum
35   passover of endogenous mFIB.

In summary, mouse no. 1/Fo (founder) produced rFIB
in milk at a level of about 30 μg/ml or greater over 3
lactations as detected by either the P/M or P/P ELISA

methods, and has passed this trait on into the F1 generation which, in mouse no. 1-11/F1, produced 32 µg/ml rFIB.

TABLE 7
Inheritability of Transgenic rhBIB Gene

Mouse No.	µg rhFIB/ml 5X diluted whey	µg rhFIB/ml milk
1/Fo	14.0 ± 2.0	71.0
1-11/F1	10.4 ± 0.8	53.0
Control whey	4.0 ± 0.7	21.0

These productions are substantially greater than the 2 µg/ml produced in the COS1 system described by Roy *et al.*, 1991, above.

Western analysis of control mouse milk using polyclonal anti-human fibrinogen antibody showed about 21 µg/ml of background fibrinogen signal (see Table 7). Western analysis that uses the clonal number 311 or Y18 monoclonal antibody has a lower limit of sensitivity of about 1 µl sample of 100 µg/ml fibrinogen (Phastgel system) or 20 µl of 25 µg/ml in a 3" by 4" SDS-PAGE gel.

WO 95/22249

PCT/US95/01944

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TABLE 6

	A	B	C	D	E	WHEY	MILK
	Animal	Dilutions	AVG ABS 490nn		FIB µ/ml	AVG FIB µ/ml	AVG FIB µ/ml
1							
2	#1 DAY 6	100	0.623	0.132	13.2		
3		200	0.523	0.088	17.6	16.3	27.6
4		400	0.434	0.049	19.4		
5		200	0.334	0.018	14.7		
6							
7	#22	100	0.374	0.022	2.16		
8		200	0.335	0.018	3.69	3.86	6.56
9		400	0.273	0.014	5.73		
10		800	0.251	0.013	10.3		
11							
12	#23	400	0.477	0.067	6.75		
13		200	0.384	0.026	5.22	7.55	12.8
14		400	0.338	0.013	7.44		
15		600	0.261	0.013	10.8		

SUBSTITUTE SHEET (RULE 26)

33

SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT:
        (A) ADDRESSEE: American National Red Cross
        (B) STREET: 1730 E Street, N.W.
        (C) CITY: Washington, D.C.
        (E) COUNTRY: USA
        (F) POSTAL CODE: 20006

    (i) APPLICANT:
        (A) ADDRESSEE: Virginia Tech Intellectual Properties, Inc.
        (B) STREET: 1900 Kraft Drive
        (C) CITY: Blacksburg
        (D) STATE OR PROVINCE: Virginia
        (E) COUNTRY: USA
        (F) POSTAL CODE: 24060

    (i) APPLICANT:
        (A) ADDRESSEE: University of North Carolina,
                    Office of Research Services
        (B) STREET: Campus Box 4100
        (C) CITY: Chapel Hill
        (D) STATE OR PROVINCE: North Carolina
        (E) COUNTRY: USA
        (F) POSTAL CODE: 27599

    (ii) TITLE OF INVENTION: TRANSGENIC FIBRINOGEN

   (iii) NUMBER OF SEQUENCES: 12

    (iv) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCCCAGCTGC AGCCATGAAG   20

(2) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 28 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

34

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CCCCAGGGAT ATCCCTGCAG CCATGAAG   28

(2) INFORMATION FOR SEQ ID NO:3:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GCTAGGTACC ATGTTTTCCA TGAGGATCGT   30

(2) INFORMATION FOR SEQ ID NO:4:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (iv) ANTI-SENSE: YES

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GATTTAGAGC GGGACAGATC CCATGGTGAC   30

(2) INFORMATION FOR SEQ ID NO:5:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCTAGGTACC ATGAAAAGAA TGGTTTCGTG   30

(2) INFORMATION FOR SEQ ID NO:6:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (iv) ANTI-SENSE: YES

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGAAGAAGGG TGTCGTTATC CCATGGTGAC   30

(2) INFORMATION FOR SEQ ID NO:7:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GCTAGGTACC ATGAGTTGGT CCTTGCACCC   30

(2) INFORMATION FOR SEQ ID NO:8:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (iv) ANTI-SENSE: YES

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TTGTCCGACC TCTGCAAATT CCATGGTGAC   30

(2) INFORMATION FOR SEQ ID NO:9:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 25 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CTGTGTGGCC AAGAAGGAAG TGTTG   25

(2) INFORMATION FOR SEQ ID NO:10:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 23 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

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CCCGATAGCC ACCTCCTCTG ATG   23

(2) INFORMATION FOR SEQ ID NO:11:

  (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 23 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

  (ii) MOLECULE TYPE: DNA (genomic)

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CCCGATAGCC ACCTCCTCTG ATG   23

(2) INFORMATION FOR SEQ ID NO:12:

  (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 24 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

  (ii) MOLECULE TYPE: DNA (genomic)

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CCTGGACTTC AAAGTAGCAG CGTC   24

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WHAT IS CLAIMED IS:

1.   A transgenic non-human animal expressing a heterologous recombinant fibrinogen protein and secreting said fibrinogen protein into a body fluid of said animal.

2.   An animal of claim 1, wherein said animal has stably integrated in its genome a heterologous fibrinogen subunit chain-encoding DNA sequence selected from the group consisting of Aα, Bβ and Gγ DNA encoding the three subunit chain polypeptides of said fibrinogen, and wherein each said heterologous DNA sequence is operably linked to a *cis*-acting expression promoter-containing regulatory sequence and to a sequence encoding a transmembrane secretory signal peptide for secretion of said fibrinogen into said body fluid.

3.   An animal of claim 2, wherein said promoter is a whey acid protein promoter.

4.   An animal of claim 2, wherein said promoter is a casein promoter.

5.   An animal of claim 2, wherein said promoter is a β-lactoglobulin promoter.

6.   An animal of claim 2, wherein said promoter is an α-lactalbumin promoter.

7.   An animal of claim 1, wherein said fibrinogen is human fibrinogen.

8.   An animal of claim 1, wherein said fibrinogen is non-human animal fibrinogen.

9.   An animal of claim 1, wherein said fibrinogen protein comprises a fibrinogen subunit chain polypeptide.

10.   An animal of claim 1, wherein said fibrinogen protein comprises a modified fibrinogen molecule.

11.   An animal of claim 1, wherein said animal is a rodent, rabbit, cat, dog, pig, sheep, goat, cow or horse.

12.   An animal of claim 1, wherein said fibrinogen protein is synthesized as a fusion protein with a non-fibrinogen protein.

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13. An animal of claim 12, wherein said non-fibrinogen protein is a milk protein selected from the group consisting of whey acid protein, casein, α-lactalbumin and β-lactoglobulin.

14. A process for producing a heterologous recombinant fibrinogen protein, comprising the steps of:

a) providing a transgenic, non-human animal within whose genome is stably integrated a heterologous DNA sequence encoding a fibrinogen subunit chain selected from the group consisting of Aα, Bβ and Gγ, wherein each said heterologous DNA encodes a different fibrinogen subunit chain polypeptide, and wherein each said heterologous DNA sequence further includes a *cis*-acting, expression promoter-containing regulatory sequence operably linked to said heterologous DNA sequence and a transmembrane secretory signal peptide, said regulatory sequence being specifically active in a mammary gland of said animal and said signal peptide being capable of directing the secretion of expressed fibrinogen into a body fluid of said animal;

b) collecting said body fluid; and

c) isolating said expressed fibrinogen protein from said body fluid.

15. A process of claim 14, wherein said promoter is a whey acid protein promoter.

16. A process of claim 14, wherein said promoter is a casein promoter.

17. A process of claim 14, wherein said promoter is a β-lactoglobulin promoter.

18. A process of claim 14, wherein said promoter is an α-lactoglobulin promoter.

19. A process of claim 14, wherein said fibrinogen protein is synthesized as a fusion protein with a non-fibrinogen protein.

20. A process of claim 19, wherein said non-fibrinogen protein is a milk protein selected from

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the group consisting of whey acid protein, casein, γ-lactalbumin and ß-lactoglobulin.

21. A process of claim 14, wherein said transgenic animal is a rodent, rabbit, cat, dog. pig, sheep, goat, cow or horse.

22. A process of claim 14, wherein said fibrinogen protein is human fibrinogen.

23. A process of claim 14, wherein said fibrinogen protein is rodent, rabbit, cat, dog, pig, sheep, goat, cow or horse fibrinogen protein.

24. A process of claim 14 wherein said body fluid is milk.

25. A process of claim 14, wherein said body fluid is blood.

26. A process of claim 14, wherein said body fluid is urine.

27. A process of claim 14, wherein said fibrinogen protein produced is a fibrinogen subunit chain polypeptide.

28. A process of Claim 14, wherein said fibrinogen protein comprises a modified fibrinogen molecule.

29. A body fluid from a transgenic non-human animal, wherein said animal has stably incorporated into its genome a heterologous DNA sequence encoding a fibrinogen subunit chain protein selected from the group consisting of Aα, Bß and Gγ subunit chain proteins.

30. Plasmid pUCWAP4 (ATCC Accession No. _____).

31. Plasmid pUCWAP5 (ATCC Accession No. _____).

32. Plasmid pUCWAP5/FIB Aα (ATCC Accession No. _____).

33. Plasmid pUCWAP5/FIB Bß1 (ATCC Accession No. _____).

34. Plasmid pUCWAP5/FIB Gγ1 (ATCC Accession No. _____).

35. A prokaryotic cell transformed by a plasmid selected from any one of Claims 32 to 34, inclusive.

FIG. 1

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FIG. 2

FIG. 3

FIG. 4

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FIG. 5
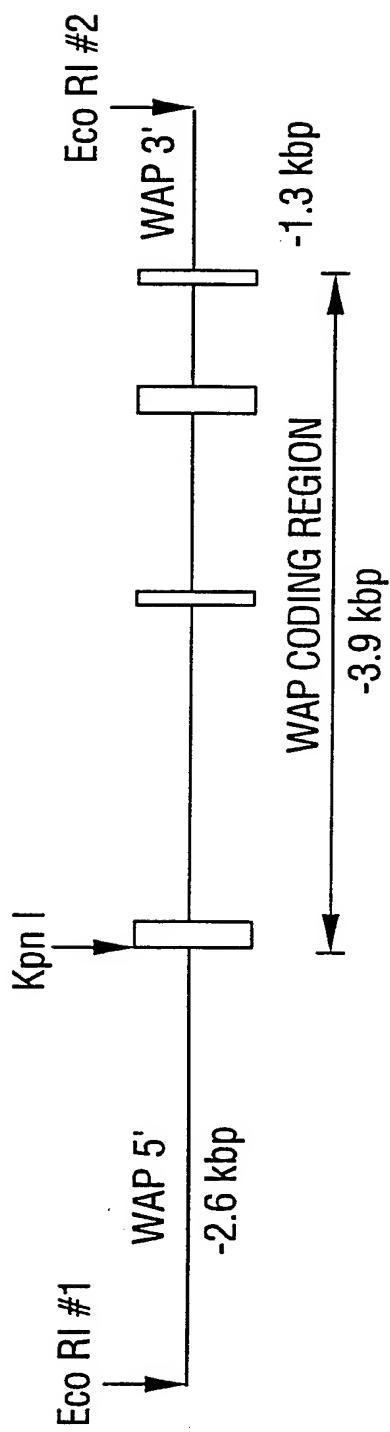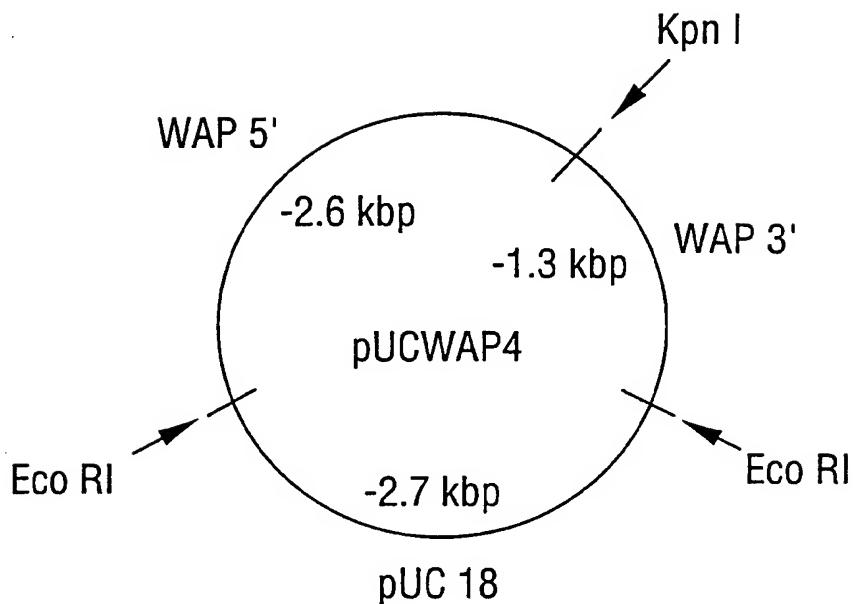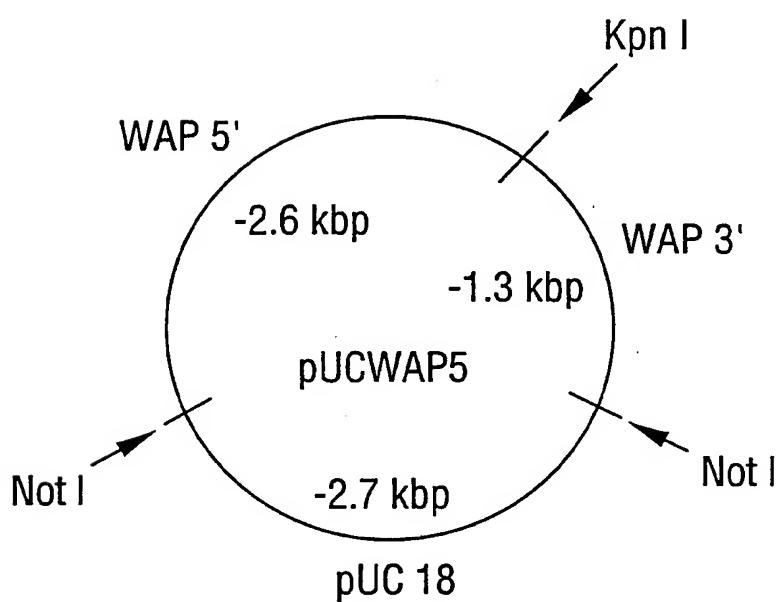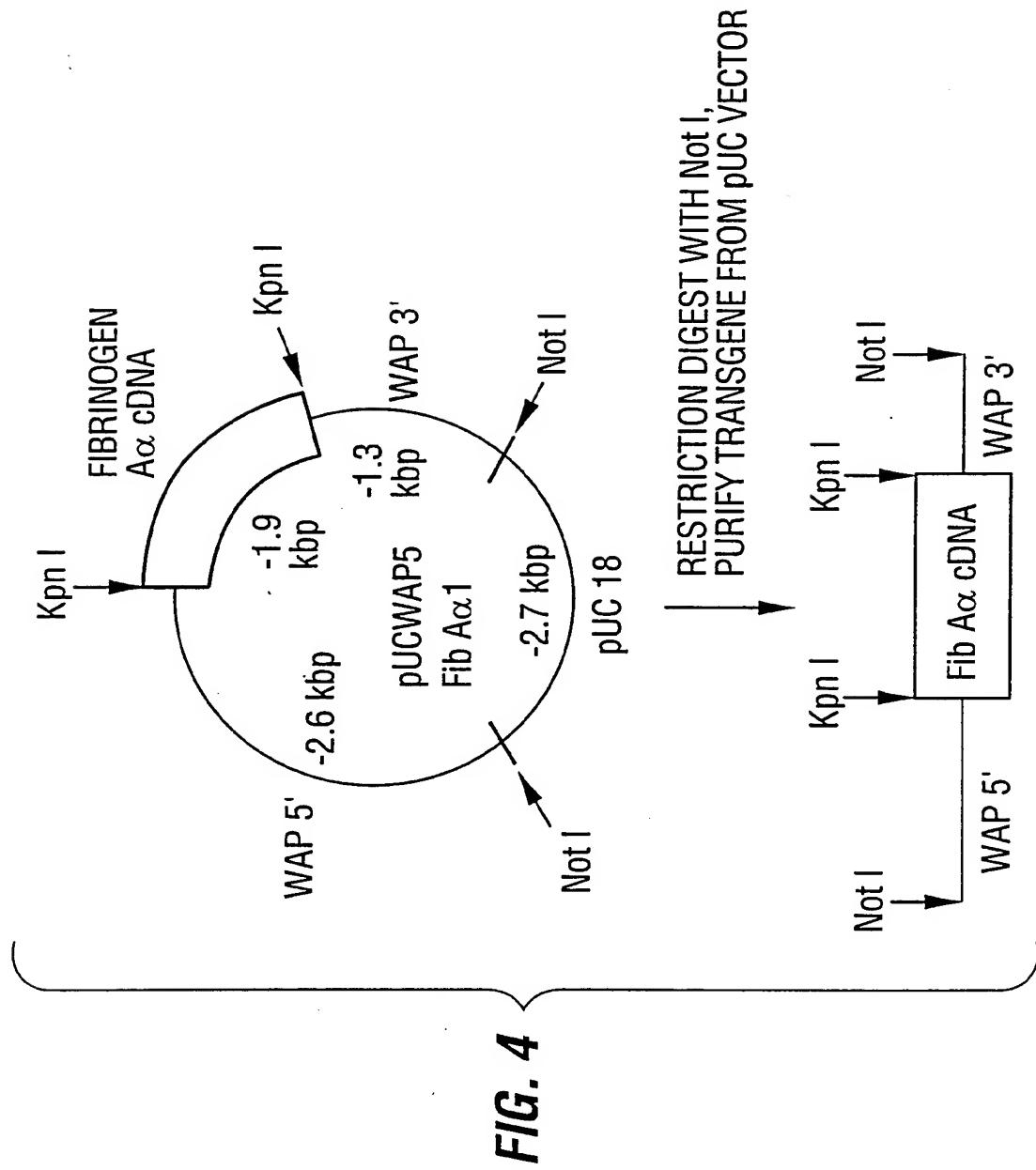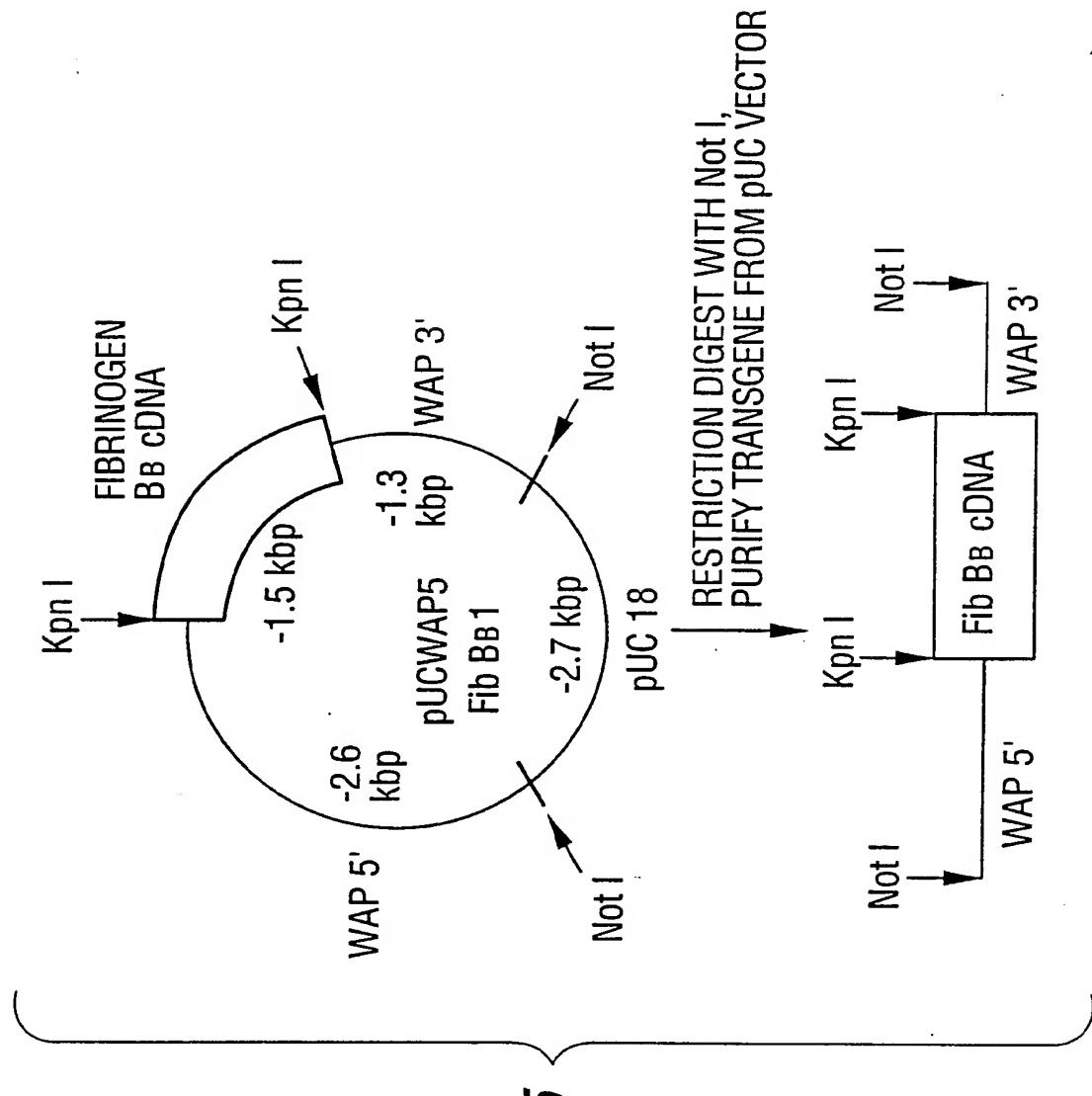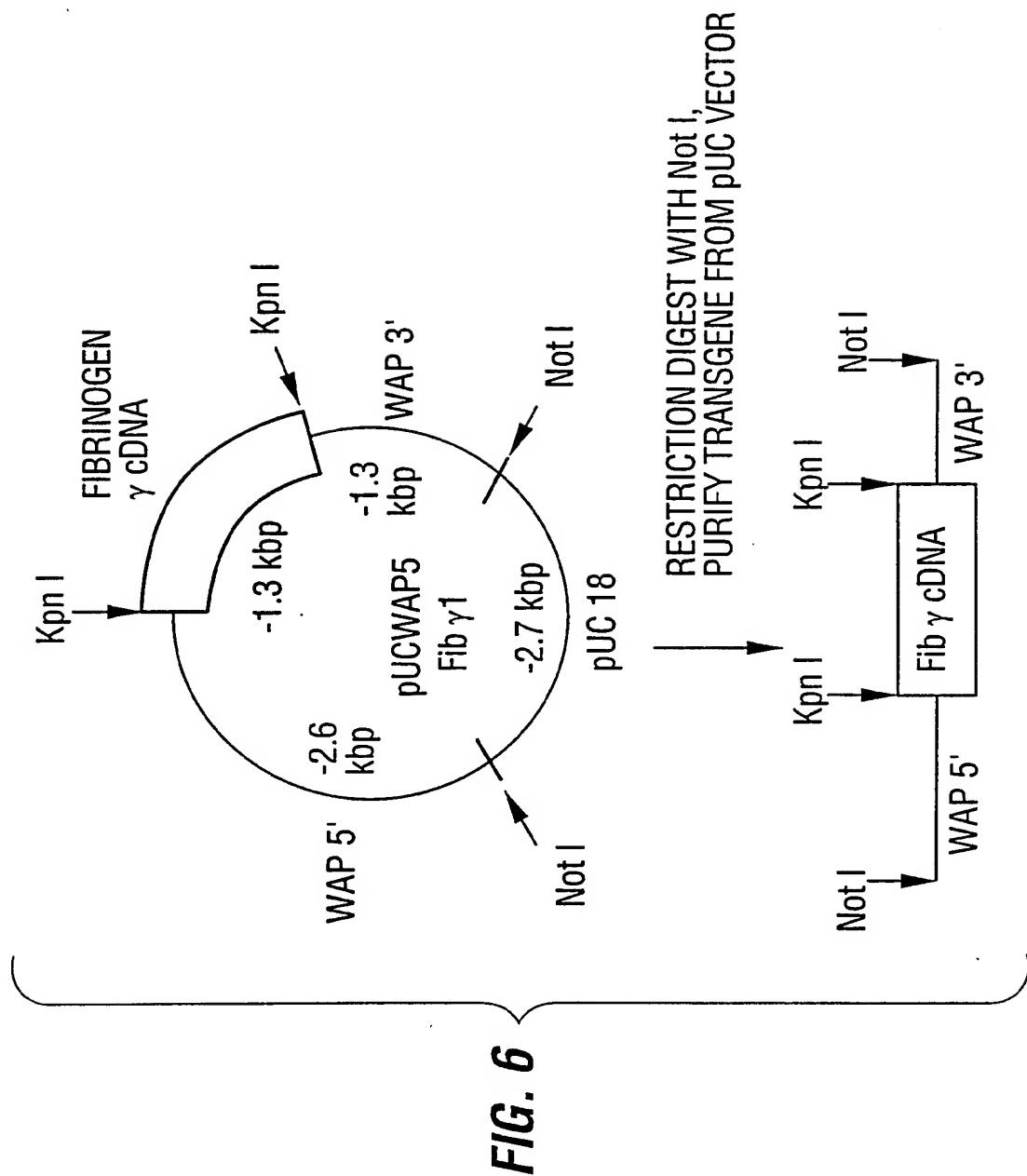
FIG. 6

FIG. 7

FIBRINOGEN FAMILY TREE
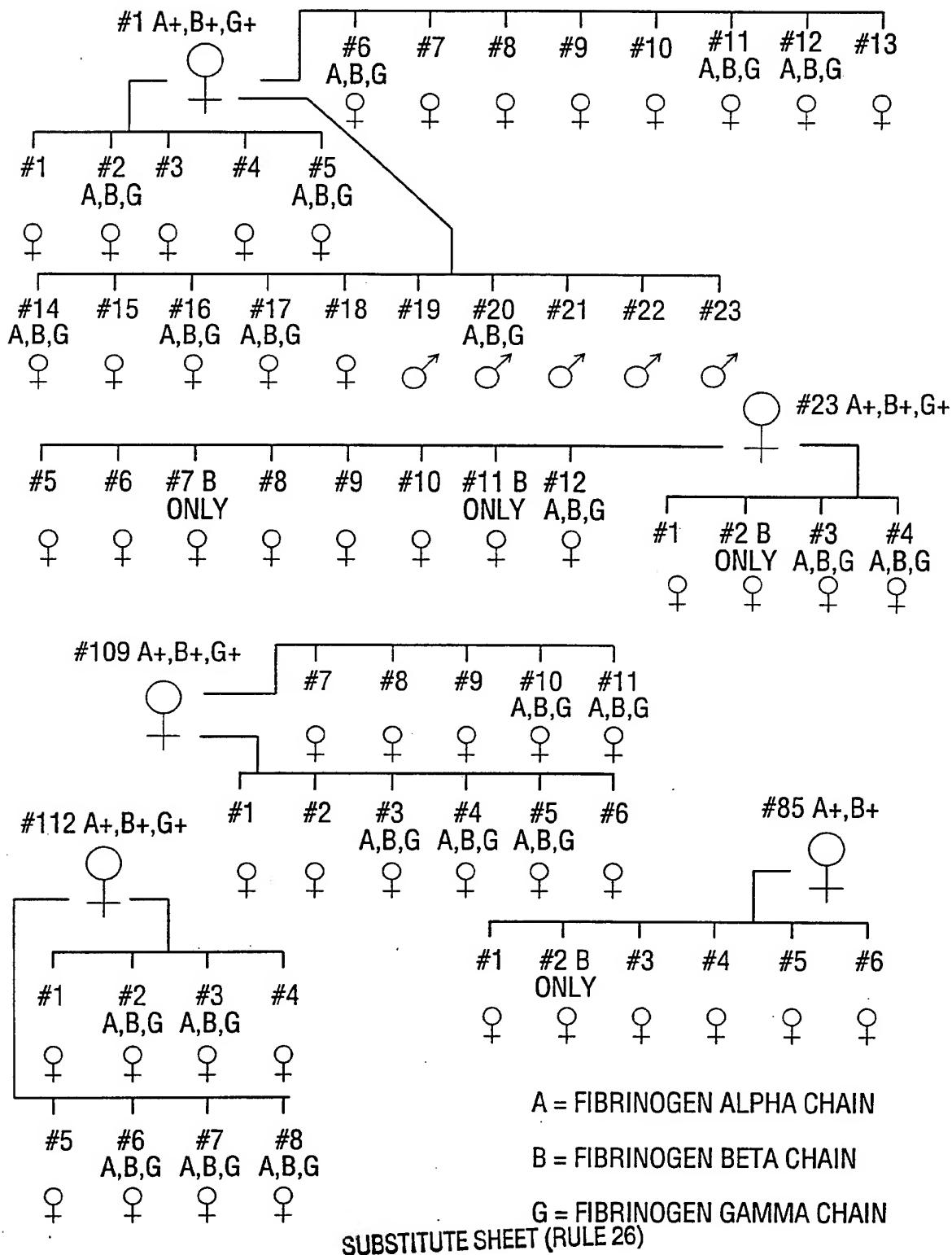
A = FIBRINOGEN ALPHA CHAIN

B = FIBRINOGEN BETA CHAIN

G = FIBRINOGEN GAMMA CHAIN

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   A01K67/027   C12N15/63

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   C12N   A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	FIBRINOLYSIS, vol. 8, no. 1, 1994 page 102 D. PRUNKARD ET AL. 'Expression of recombinant human fibrinogen in the milk of transgenic mice' *see the abstract 285*	1-35
Y	WO-A-92 11757 (AMERICAN RED CROSS) 23 July 1992 *see the whole patent*	1-35
	-/--	

| X | Further documents are listed in the continuation of box C. | | X | Patent family members are listed in annex. |

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search	Date of mailing of the international search report
28 June 1995	0 4. 08. 95

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF CONTROLLED RELEASE, vol. 29, 1994 pages 213-221, S.H. LEE ET AL. 'Production of biomedical proteins in the milk of transgenic dairy cows: the state of the art' *see the whole article*	1-35
Y	BLOOD COAGULATION AND FIBRINOLYSIS, vol. 4, 1993 pages 55-59, S.T. LORD ET AL. 'Purification and characterization of recombinant human fibrinogen' *see the whole article*	1-35
Y	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 266, no. 8, 15 March 1991 pages 4758-4763, S. N. ROY ET AL. 'Assembly and secretion of recombinant human fibrinogen' *see the whole article*	1-35
Y	BIOCHEMISTRY, vol. 22, 1983 pages 3237-3244, M.W. RIXON ET AL. 'Characterization of a complementary DNA coding for the alfa chain of human fibrinogen' *see the whole article*	1-35
Y	BIOCHEMISTRY, vol. 22, 1983 pages 3250-3256, D.W. CHUNG ET AL. 'Characterization of a cDNA coding for the gamma chain of human fibrinogen' *see the whole article*	1-35
Y	BIOCHEMISTRY, vol. 22, 1983 pages 3244-3250, D.W. CHUNG ET AL. 'Characterisation of a cDNA coding for the beta chain of human fibrinogen' *see the whole article*	1-35

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9211757	23-07-92	AU-A-	1228592	17-08-92
		EP-A-	0591219	13-04-94
		JP-T-	6507307	25-08-94